

A newly identified lncRNA MAR1 acts as a miR-487b sponge to promote skeletal muscle differentiation and regeneration

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Abstract

Background Skeletal muscle atrophy induced by either aging (sarcopenia) or mechanical unloading is associated with serious health consequences. Long non-coding RNAs (lncRNAs) are implicated as important regulators in numerous physiological and pathological processes.

Methods Microarray analysis was performed to identify the differentially expressed lncRNAs in skeletal muscle between adult and aged mice. The most decreased lncRNA in aged skeletal muscle was identified. The C2C12 mouse myoblast cells were used to assess the biological function of the lncRNA *in vitro*. The target microRNA of lncRNA and the target protein of microRNA were predicted by bioinformatics analysis and validated *in vitro*. Furthermore, the biology function of the lncRNA *in vivo* was investigated by local overexpression or knockdown the lncRNA in skeletal muscle. The therapeutic effect of the lncRNA overexpression in age-related or mechanical unloading-induced muscle atrophy was also evaluated.

Results We identified a novel lncRNA (muscle anabolic regulator 1, MAR1) which was highly expressed in mice skeletal muscle and positively correlated with muscle differentiation and growth *in vitro* and *in vivo*. We predicted and validated that microRNA-487b (miR-487b) was a direct target of MAR1. We also predicted and validated that Wnt5a, an important regulator during myogenesis, was a target of miR-487b in C2C12 cells. Our findings further demonstrated that enforced MAR1 expression in myoblasts led to derepression of Wnt5a. Moreover, MAR1 promoted skeletal muscle mass/strength and Wnt5a protein level in mice. Enforced MAR1 expression in mice attenuated muscle atrophy induced by either aging or unloading.

Conclusions The newly identified lncRNA MAR1 acts as a miR-487b sponge to regulate Wnt5a protein, resulting in promoting muscle differentiation and regeneration. MAR1 could be a novel therapeutic target for treating muscle atrophy induced by either aging or mechanical unloading.

Keywords Long non-coding RNA; miR-487b; Wnt5a; Muscle differentiation; Muscle regeneration

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Introduction

Skeletal muscle undergoes remarkable adaptations in response to environmental stimuli.¹ Numerous conditions, including prolonged periods of muscle inactivity² and aging,³ induce skeletal muscle atrophy. Skeletal muscle atrophy results in muscular weakness and diminished quality of life, which also increases morbidity and mortality.⁴

The skeletal muscle differentiation is influenced by multiple signalling pathways. The myogenic regulatory factors (MRFs), including MyoD, Myf5, and myogenin, are the core components of the myogenic pathway.⁵ These MRFs, together with their co-regulator, myocyte enhancer factor 2C (MEF2C),⁶ play important roles during myogenesis.⁷ Wingless-type (Wnt) signals control the expression of MRFs.⁸ Wnt5a, a member of Wnt family, regulates MyoD and Myf5 during myogenesis.⁹ However, the molecular mechanism of skeletal muscle atrophy still needs to be extensively explored.

It has been reported that long non-coding RNAs (lncRNAs) (>200 nucleotides), including H19 (gene comes from colon pH19),^{10,11} linc-MD1 (long intergenic noncoding RNA),¹² Yam-1 (YY1-associated muscle lincRNA),¹³ Malat1 (metastasis-associated lung adenocarcinoma transcript 1),¹⁴ and linc-mg (myogenesis-associated lincRNA),¹⁵ are associated with muscle differentiation. However, these studies mainly focus on the physiological function of lncRNAs in the cellular level, the pathological roles of lncRNAs in skeletal muscle atrophy, especially induced by either aging or mechanical unloading, are still unknown. Thus, the aim of current study is to explore the role of lncRNA in regulating muscle differentiation during the skeletal muscle atrophy induced by aging or mechanical unloading.

In the present study, we identified a novel lncRNA [muscle anabolic regulator 1 (MAR1)] which was positively correlated with muscle differentiation and growth *in vitro* and *in vivo*. We predicted the target microRNA of MAR1 by bioinformatics analysis and validated it *in vitro*. Moreover, the effect of skeletal muscle-specific overexpression of MAR1 on skeletal muscle atrophy induced by either aging or unloading was investigated. This study could provide a novel strategy for the treatment of muscle atrophy.

Materials and methods

Animals

Two-month-old, six-month-old, twenty-two-month-old, and twenty-four-month-old C57BL/6J mice were used. All the animals were maintained under standard animal housing conditions (12-h light, 12-h dark cycles, and free access to food and water). All the experimental procedures were approved by the Committees of Animal Ethics and Experimental Safety of the Chinese University of Hong Kong.

Hindlimb suspension procedure

Six-month-old mice were subjected to hindlimb suspension (HS) for 28 days following established procedure.^{16,17} Briefly, a strip of adhesive tape was applied to the animal's tail, which was suspended by passing the tape through a fish-line swivel that was attached to a metal bar on the top of the cage. The forelimbs were allowed to touch the grid floor and the animals could move around the cage for free access to food and water. The suspension height was adjusted to prevent the hindlimbs from touching any supporting surface while maintaining a suspension angle of approximately 30°. Distal tip of the tail was examined to ensure that the procedure did not occlude blood flow to the tail.

Cell culture

The C2C12 mouse myoblast cell line was supplied by American Type Culture Collection. Cells were cultured at subconfluent densities in growth medium, made up of Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin. C2C12 myoblast cells were differentiated into myocytes or myotubes in differentiation medium, consisting of DMEM containing 2% heat-inactivated horse serum, and 1% penicillin/streptomycin. All these cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.¹⁸

Isolation of total RNA and real-time PCR analysis

Total RNA from cell line and tissue samples was isolated by Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA synthesis for mRNA and lncRNA detection were carried out using SuperScript III first strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). The Fast start Universal SYBR Green Master (Roche, Indianapolis, IN, USA) was applied for the quantitative RT-PCR. Quantification of amplicons was done using ABI 7300 software (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as endogenous controls for normalization. All the primer sequences were listed in Table S1. The relative fold changes of candidate genes were analysed by using 2^{-ΔΔCT} method.¹⁸

Microarray analysis

Gastrocnemius muscles from three 6-month-old and three 24-month-old mice were collected. Sample preparation and microarray hybridization were performed by Kangchen Bio-tech, Shanghai P.R. China.¹⁹ Briefly, RNA was purified

from 1 µg total RNA after removal of rRNA (mRNA-ONLY Eukaryotic mRNA Isolation Kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method. The labelled cRNAs were hybridized onto the Mice LncRNA Array v2.0 (8 × 60 K, Arraystar, Rockville, MD, USA). After having washed the slides, the arrays were scanned by the Agilent Scanner G2505B (Agilent Technologies, Santa Clara, CA, USA). Agilent Feature Extraction software (version 10.7.3.1) was utilized to analyse acquired array images. Quantile normalization and subsequent data processing were carried out using the GeneSpring GX v11.5.1 software package (Agilent Technologies, Santa Clara, CA, USA). Differentially expressed lncRNAs was identified through fold change filtering (Fold Change ≥ 2.0 or ≤ 0.5), paired *t*-test ($P < 0.05$) and multiple hypothesis testing (FDR < 0.05). *P* values and FDR were calculated by Microsoft Excel and MATLAB, respectively.

Lentiviral vector construction and lentivirus production

For the construction of MAR1-overexpression lentiviral vector, mouse α -skeletal actin promoter (2.0 kb) and full-length of MAR1 (641 bp) were subcloned into the lentiviral GV112 vector which provided by Shanghai Genechem Co., Ltd. (Shanghai, China) according to the manufacturer's instruction.²⁰ For the MAR1-knockdown lentiviral vector, shRNA targeting MAR1 or negative control scramble sequence were subcloned into the GV112 vector, respectively. Three shRNA sequences were designed by Shanghai Genechem.ltd (shMAR1-1: 5'-AGCAATCCAATTCATGTTTAA-3', shMAR1-2: 5'-GTGTTATCTGGTTCATATCAAG-3' and shMAR1-3: 5'-TTCATGGTTTTTAA AAAAATGT-3'). For the production of lentivirus, the expression vectors were co-transfected with packaging plasmid pHelper 1.0 vector (Shanghai Genechem Co., Ltd.) and envelope plasmid pHelper 2.0 vector (Shanghai Genechem Co., Ltd.) into 293T cells using TransIT-LT1 (Mirus Bio). The supernatant was collected 48 and 72 h post-transfection, concentrated by ultracentrifugation at 25 000 r.p.m. for 90 min and resuspended in an appropriate volume of OptiMEM (Gibco, Waltham, MA, USA). The infectious particles titre (IU/mL) was determined by quantitative real-time PCR genomic DNA of transduced cells as described elsewhere.²¹

Cell infection

Based on previously reported protocol with modification,²² C2C12 mouse myoblasts were seeded in 6-well plates and cultured until 60% confluent. Medium was then removed and 1.5 × 10⁸ IU viral particles were added together with 8 µg/mL hexadimethrine bromide (Sigma-Aldrich, St. Louis, MO, USA). The medium volume was made up to 500 µl with

DMEM and then incubated at 37°C and 5% CO₂ for 8 h, at which time the DMEM plus viral particles was changed to DMEM with 10% fibre channel standard and the cells were cultured for 1–7 days.

Bioinformatics analysis for targeted gene prediction

The corresponding targeted genes for MAR1 and miR-487b were predicted using RNAhybrid 2.12 (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>). RNAhybrid is a tool used for predicting biological targets of certain RNA by searching for the presence of conserved sites that match seed region of a RNA.²³

Luciferase reporter assays

Following previous established procedure, the putative sequences of the binding site in MAR1 and the mutated sequences were cloned into a pmirGLO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to form the reporter vector.²⁴ The report vector was co-transfected with miR-487b-WT or miR-487b-Mut into C2C12 cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) after 48 h transfection for luciferase assay using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol. Similarly, the putative sequences of the binding site in miR-487b for Wnt5a and the mutated sequences were cloned into a pmirGLO Dual-luciferase miRNA Target Expression Vector to form the reporter vector. The report vector was co-transfected with Wnt5a 3' UTR-WT or Wnt5a 3' UTR-Mut into C2C12 cells by using Lipofectamine 2000. The measurement of Luciferase activities was handled similarly as described above.

Biotin-labelled MAR1 pulled down

Biotin-labelled MAR1 was synthesized by Sangon Biotech. (Shanghai, China). Different doses of biotin-labelled MAR1 (0.5, 5, and 50 mM) were incubated with cytoplasmic lysates from miR-487b transfected C2C12 cells for 30 min at room temperature, and complexes were isolated with streptavidin-coated magnetic Dynabeads (Dyna, Waltham, MA, USA). After wash steps, the captured RNA was purified and analysed with real-time PCR.²⁵

Western blotting

According to previous established procedures, cells and muscle samples were harvested, washed with 1× phosphate-buffered saline (PBS), and lysed in NP40 lysis buffer (50 mM

Tris-HCl, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 10% glycerol) with protease inhibitors cocktail (Sigma-Aldrich, St. Louis, MO, USA).¹⁸ Proteins were separated in SDS-PAGE, transferred, and immunoblotted with various antibodies. The antibodies used were anti-Wnt5a (1:1500; Invitrogen, Carlsbad, CA, USA), anti-MyoD (1:1500; Invitrogen), anti-MyoG (1:1500; Invitrogen), anti-MEF2C (1:1500; Invitrogen), anti-Myf5 (1:1500; Invitrogen), and anti- β -actin (1:3000; Santa Cruz Biotechnology, Dallas, TX, USA).

In vivo lentiviral particle administration

Following established protocols with minor modifications, lentiviral particle for MAR1 overexpression or knockdown was injected into the mid-portion of one side GA muscles of each mouse at dosage of 10.0×10^8 IU/mL.²⁶ Total 15 μ l of viral preparation was injected into each muscle. For the adult and aged mice ($n = 10$), the mice were sacrificed 2 months after lentivirus administration. For the mechanical unloading mice ($n = 10$), the mice were sacrificed after 28-day HS. The specific force generated by gastrocnemius muscle was evaluated by *in situ* muscle functional testing before sacrifice. The gastrocnemius muscles were collected and weighed. The muscles were cryosectioned for immunofluorescent staining of dystrophin. The mean muscle fibre cross-sectional area (CSA) was determined. MAR1 level and mRNA levels of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) were assessed by real-time PCR. The protein levels of Wnt5a and above myogenic markers were evaluated by western blot analysis.

In situ muscle functional testing

In situ muscle functional testing was performed following the established protocol.²⁷ The animals were anaesthetised with isophorone gas inhale system and then placed on a 37°C heated platform. The gastrocnemius muscle was exposed and the distal tendon was attached to the lever arm of a position feedback motor. The knee joint was immobilized by clamping it to the platform (*In Vivo* Muscle Test system 1300A, Aurora Scientific Inc., Aurora, Canada). Needle electrode was inserted through the skin and positioned on the peroneal nerve to stimulate contraction of gastrocnemius muscle. The twitch force and peak isometric force at 150 Hz for 700 ms were recorded and the specific force was calculated with the ASI Dynamic Muscle Control Software (DMC v5.1 beta, Aurora Scientific Inc.).

Immunofluorescent staining

The dissected gastrocnemius muscles were frozen in liquid nitrogen-cooled isopentane and then embedded in OCT medium. Serial cross-sections (6 μ m thickness) were cut

from the mid-belly of the muscles on a cryostat at -20°C for immunofluorescent staining. The sections were fixed in cold acetone (4°C) for 10 min and then incubated for 60 min in a 1% bovine serum albumin (BSA)/PBS solution to block non-specific binding. The sections were then incubated overnight at 4°C with antibody cocktail of primary antibodies against dystrophin (1:100, Santa Cruz Biotechnology, Dallas, TX, USA). Following three washes in PBS, the sections were incubated with secondary antibody (1:500, Santa Cruz Biotechnology) for 60 min. The sections were again washed in PBS and mounted in Mowiol mounting medium (Merck KGaA, Darmstadt, Germany). Slides were visualized with an Axio Observer Z1 microscope (Carl Zeiss, Jena, Germany) using conventional wide-field fluorescence microscopy as well as optical sectioning via structured-illumination fluorescence microscopy (Apotome, Carl Zeiss).

Muscle fibre cross-sectional area (CSA) calculation: Six images of different locations in one section were captured at 200 \times magnification for muscle fibre CSA calculation. Fifty contiguous myofibers in each image were circled to obtain an average of 300 fibres for each muscle. Image J software (National Institutes of Health, Bethesda, MD, USA) were used to determine the area and number of muscle fibres. The mean muscle fibre CSA (μm^2) of each muscle was obtained by dividing the total area of circled muscle fibres in six images by the total fibre number in circled area of six images. The mean muscle fibre CSA in each group was further calculated and normalized to Control or Baseline group^{28,29} (Supplementary Figure 1).

Statistical analysis

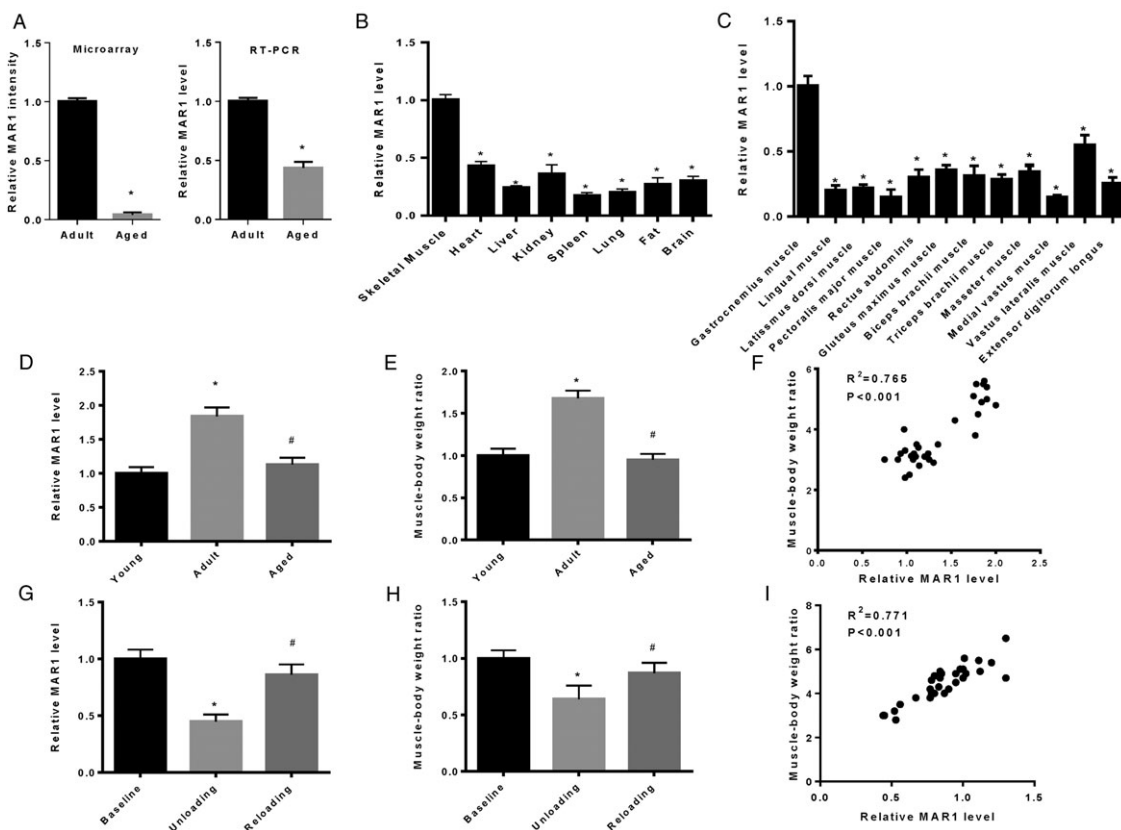
Data are presented as means \pm SEM. Each separate sample was analysed in triplicate to yield an average value, n = number of samples in each group (3, 5, and 10 for microarray analysis, *in vitro* evaluation and *in vivo* evaluation, respectively). GraphPad Prism v. 6.0 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis of the data. All results between groups were analysed by applying the one-way analysis of variance (ANOVA) and Student's *t*-test. A difference with $P < 0.05$ was regarded as statistical significance.

Results

Positive correlation of lncRNA MAR1 expression with muscle mass during aging and mechanical unloading in mice

We utilized lncRNA microarray to compare the differentially expressed lncRNAs in gastrocnemius muscle between adult

Figure 1 Expression level of muscle anabolic regulator 1 (MAR1) in gastrocnemius muscle associated with age-related or mechanical loading-related changes in skeletal muscle mass of mouse (A) microarray (left) and RT-PCR analysis (right) of MAR1 levels between adult and aged skeletal muscle. * $P < 0.05$ vs. adult. (B) Real-time PCR analysis of MAR1 levels in different tissue/organs of mouse. U6 is used as internal control. * $P < 0.05$ vs. skeletal muscle. (C) Real-time PCR analysis of MAR1 levels in different skeletal muscle of mouse. U6 is used as internal control. * $P < 0.05$ vs. gastrocnemius muscle. (D–F) Expression level of MAR1 (D), muscle weight of gastrocnemius/body weight ratio (M/B ratio) (E) and correlation analysis between MAR1 level and M/B ratio, (F) of gastrocnemius muscle from young (2-month-old), adult (6-month-old), and aged (24-month-old) mice. * $P < 0.05$ vs. young. # $P < 0.05$ vs. adult. (G–I) Expression level of MAR1 (G), M/B ratio (H) and correlation analysis between MAR1 level and M/B ratio. (I) of gastrocnemius muscle in adult mice with 28-day hindlimb suspension (HS) and 28-day reloading. * $P < 0.05$ vs. baseline. # $P < 0.05$ vs. unloading. $n = 3$ for microarray and $n = 10$ for animal study. Each sample was assessed in triplicate. Data are presented as mean \pm SEM.



(6-month-old, $n = 3$) and aged mice (24-month-old, $n = 3$). A 641 nt long lncRNA (AK087875) was identified as the most down-regulated lncRNA in the gastrocnemius muscle from aged mice (Figure 1A, Supplementary Figure 2 and Supplementary Table 2). We named this lncRNA as MAR1. Further PCR showed that the expression level of MAR1 was much higher in the skeletal muscle than that in other tissue/organs in adult mice ($n = 10$) (Figure 1B). Among different skeletal muscles, MAR1 was the most abundant one in gastrocnemius muscle (Figure 1C). The MAR1 level in skeletal muscle was elevated from young (2-month-old, $n = 10$) to adult (6-month-old, $n = 10$) and then decreased from adult to aged (24-month-old, $n = 10$) (Figure 1D). Muscle weight of gastrocnemius/body weight ratio (M/B ratio) reached the highest at the adult phase and decreased when the mice were aging (Figure 1E). The correlation analysis showed that there was a positive correlation between MAR1 expression

and M/B ratio during muscle growth and aging (Figure 1F). In addition, the expression level of MAR1 in skeletal muscle was down-regulated during mechanical unloading and restored during reloading ($n = 10$) (Figure 1G). M/B ratio showed a similar change pattern with the expression level of MAR1 for mice with unloading or reloading condition (Figure 1H). The correlation analysis also showed a positive correlation between MAR1 level and M/B ratio while mechanical loading altered (Figure 1I).

MAR1 promoted myogenic differentiation of C2C12 myoblasts in vitro

The time course of changes in MAR1 lncRNA and myogenic gene expression was examined in C2C12 myoblasts during differentiation. It was found that MAR1 was increased during

myogenic differentiation of C2C12 myoblasts, which was consistent with the changes in expression levels of myogenic markers (MyoD and MyoG) (Figure 2A). To further explore the biological functions of MAR1 in C2C12 cells, we constructed a lentiviral vector encoding either MAR1 or MAR1 shRNA and lentivirus for cell infection was subsequently prepared (Figure 2B). It was found that MAR1 expression was highly enforced in C2C12 cells that were transduced with lentivirus MAR1 (Figure 2C) and shMAR1-2 achieved the highest knockdown efficiency of MAR1 in C2C12 cells (Supplementary Figure 3). Enforced MAR1 expression in C2C12 cells significantly enhanced both mRNA and protein levels of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) and formation of

myotube (Figure 2D,E), whereas MAR1 shRNA decreased the above in C2C12 cells (Figure 2F–H).

Prediction and validation of miR-487b as one of target miRNAs of MAR1 in C2C12 cells

To gain further insight into the mechanism by which MAR1 regulates muscle differentiation, we predicted microRNA-487b (miR-487b) was one of target miRNAs of MAR1 by using RNAhybrid 2.12 (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>) (Figure 3A). It was further revealed that biotin-labelled MAR1 specifically pulled down miR-487b in a

Figure 2 Muscle anabolic regulator 1 (MAR1) promoted myogenic differentiation of C2C12 myoblasts *in vitro*. (A) Real-time PCR analysis of expression levels of MAR1 and myogenic markers (MyoD and MyoG) during differentiation of C2C12 myoblasts. C2C12 myoblast cells were maintained in growth medium (GM) and differentiated into myotubes in differentiation medium (DM) for 1–7 days. * $P < 0.05$ vs. GM. (B) Schematic of lentiviral vector used to produce lentivirus for overexpression or knockdown of MAR1. (C) Real-time PCR analysis of MAR1 level in MAR1 infected C2C12 cells. (D) Real-time PCR analysis of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) mRNA levels in MAR1 infected C2C12 cells. (E) Western blot analysis of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) protein levels in MAR1 infected C2C12 cells. (F) Microscopy observation of myotube formation in MAR1 infected C2C12 cells on day 7 in DM. Scale bar = 50 μm . Black arrow: Myotube. (G) Real-time PCR analysis of MAR1 level in MAR1 shRNA infected C2C12 cells. (H) Real-time PCR analysis of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) mRNA levels in MAR1 shRNA infected C2C12 cells. (I) Western blot analysis of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) protein levels in MAR1 shRNA infected C2C12 cells. (J) Microscopy observation of myotube formation in MAR1 shRNA infected C2C12 cells on day 7 in DM. Scale bar = 50 μm . Black arrow: Myotube. $n = 5$ for each group. Each sample was assessed in triplicate. U6 small nuclear RNA is used as the internal control of lncRNA and miRNA. GAPDH is used as the control for mRNA. β -Actin is used as the internal control in western blot. Data are presented as mean \pm SEM. * $P < 0.05$ vs. control.

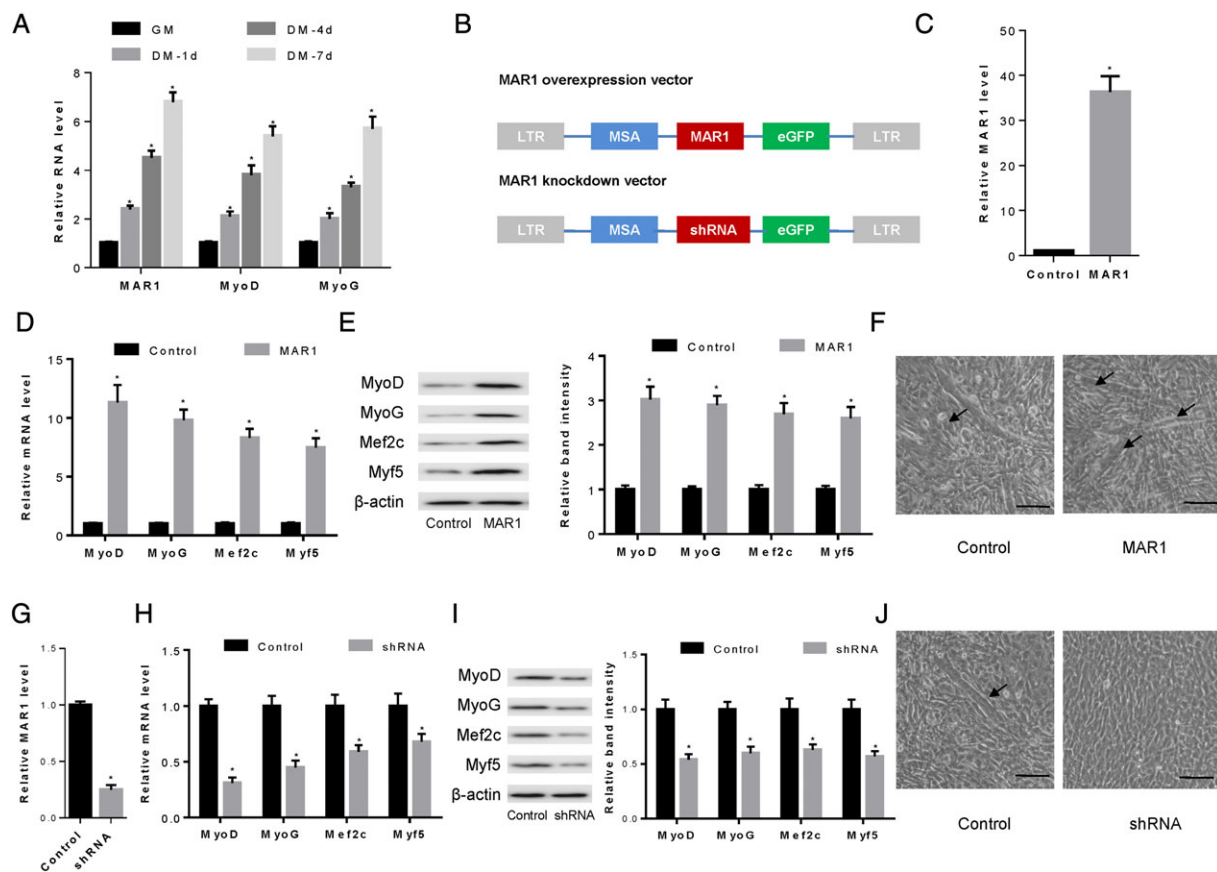
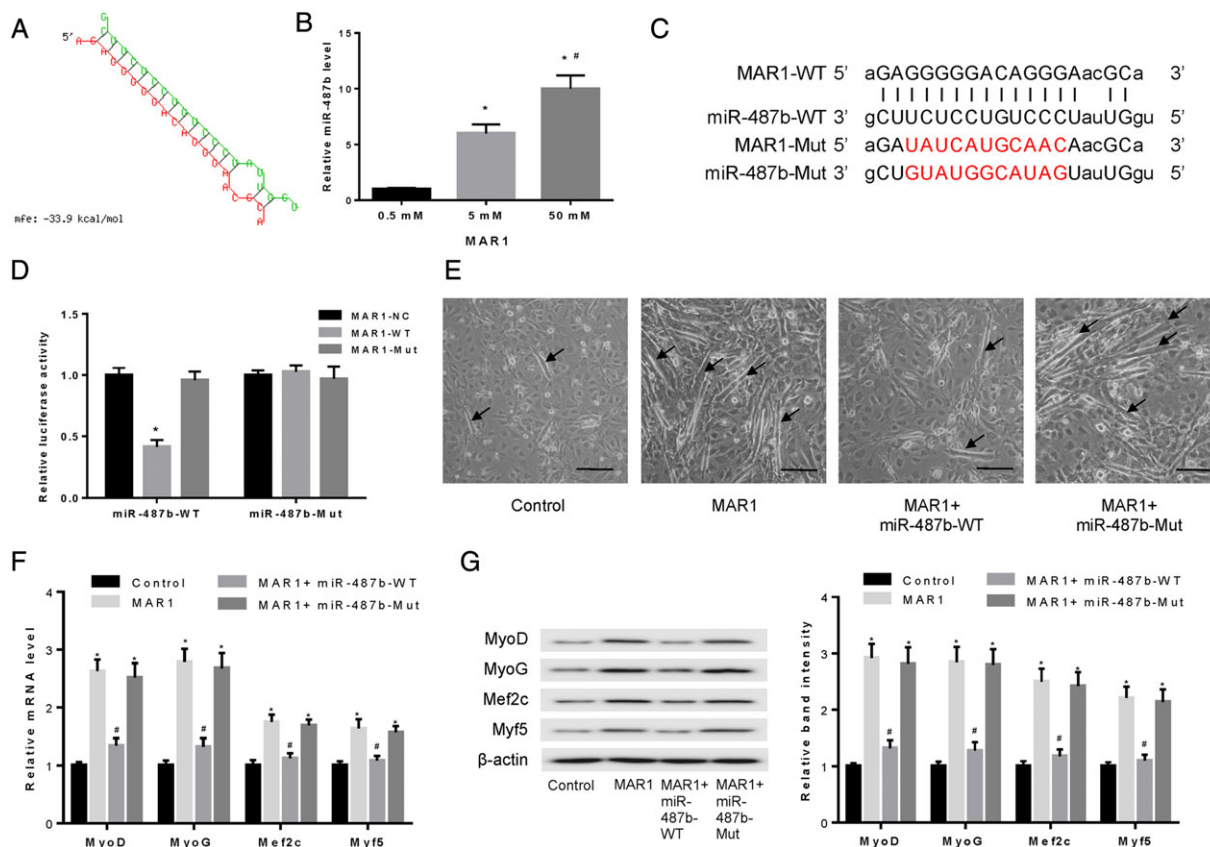


Figure 3 Prediction and validation of miR-487b as one of target binding miRNAs of MAR1 in C2C12 cells. (A) Bioinformatic prediction of miR-487b as a target miRNA of MAR1 by RNAhybrid 2.12. MFE: Minimum free energy. (B) Pull-down assay and real-time PCR analysis of miR-487b level in C2C12 cells transfected with biotin labelled MAR1 at different dosage. * $P < 0.05$ vs. 0.5 mM, # $P < 0.05$ vs. 5 mM. (C) Bioinformatic prediction of binding site of miR-487b to MAR1. (D) Luciferase reporter assay of miR-487b-WT and miR-487b-Mut in C2C12 cells transfected with either MAR1-WT or MAR1-Mut. * $P < 0.05$ vs. MAR1-NC. (E) Formation of myotube in C2C12 cells with enhanced MAR1 expression co-transfected with either miR-487b-WT or miR487b-Mut. Scale bar = 50 μ m. (F–G) Real-time PCR and western blot analysis of mRNA and protein expression of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) in C2C12 cells with enhanced MAR1 expression co-transfected with either miR-487b-WT or miR487b-Mut. * $P < 0.05$ vs. control, # $P < 0.05$ vs. MAR1. $n = 5$ for each group. Each sample was assessed in triplicate. U6 small nuclear RNA is used as the internal control of lncRNA and miRNA. GAPDH is used as the control for mRNA. β -Actin is used as the internal control in western blot. Data are presented as mean \pm SEM.



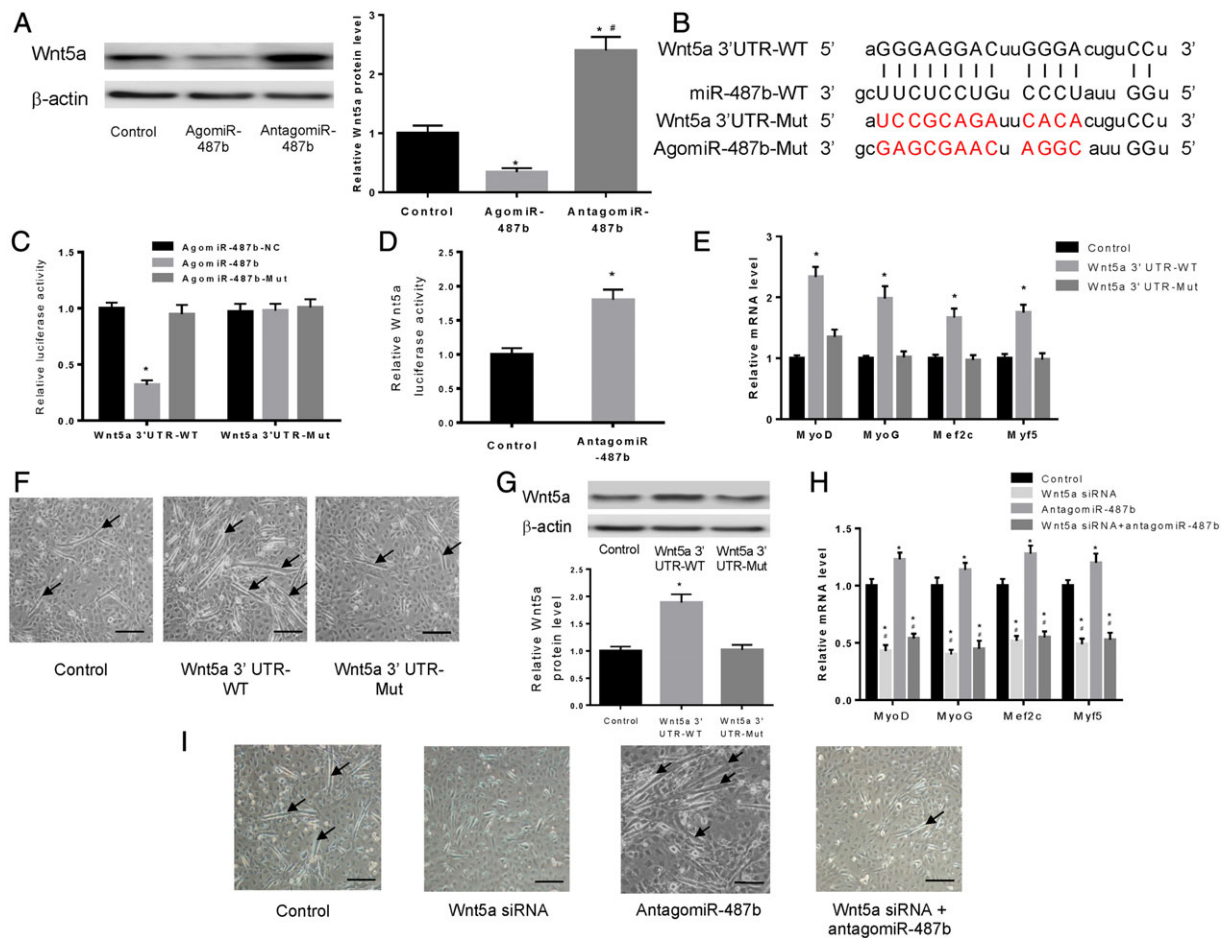
dose-dependent manner in miR-487b-transfected C2C12 cells (Figure 3B) rather than scrambled control miRNA (data not shown). In addition, the binding sites between miR-487b and MAR1 were also predicted (Figure 3C). After transfection of miR-487b mutant, we did not detect the interaction between biotin-labelled MAR1 and miR-487b mutant (data not shown). Then, we cloned the miR-487b containing either wild-type binding sites (miR-487b-WT) or mutated binding sites (miR-487b-Mut) into the downstream of luciferase reporter gene. It showed that MAR1 transfection could reduce the luciferase activity of miR-487b-WT but not affect that of miR-487b-Mut. Meanwhile, transfection of MAR1 containing mutated binding sites could not reduce the luciferase activity of either miR-487b-WT or miR-487b-Mut (Figure 3D). Furthermore, the enhanced mRNA and protein levels of myogenic markers and formation of myotube in MAR1-transfected C2C12 cells was attenuated by co-transfection with miR-487b-WT,

whereas the above findings were not observed when co-transfecting with miR-487b-Mut (Figure 3E–G).

Prediction and validation of *Wnt5a* as a target of miR-487b in C2C12 cells

Wnt5a, an important regulator during myogenesis, was reported as one of targets of miR-487b in lung cancer cells.³⁰ *Wnt5a* was down-regulated at protein level by agomiR-487b (a miR-487b agonist) in C2C12 cells, whereas antagomiR-487b (a miR-487b inhibitor) elevated the *Wnt5a* protein expression (Figure 4A). *Wnt5a* was predicted to have a miR-487b binding site in its 3' untranslated region (UTR) by RNAhybrid 2.12. We constructed the luciferase reporter gene containing either wild-type *Wnt5a* 3' UTR (*Wnt5a* 3' UTR) binding site or mutated *Wnt5a* 3' UTR

Figure 4 Prediction and validation of Wnt5a as a target of miR-487b in C2C12 cells. (A) Western blot analysis of expression level of Wnt5a in C2C12 cells treated with either agomiR-487b or antagomiR-487b. * $P < 0.05$ vs. control, # $P < 0.05$ vs. agomiR-487b. (B) Bioinformatic prediction of binding site in the 3' UTR region of Wnt5a to miR-487b. (C) Luciferase reporter assay of Wnt5a 3'UTR and Wnt5a 3' UTR-Mut in C2C12 cells transfected with either miR-487b-WT or agomiR-487b-Mut. * $P < 0.05$ vs. agomiR-487b-NC. (D) Luciferase reporter assay of Wnt5a 3' UTR in C2C12 cells treated with antagomiR-487b. (E–F) Real-time PCR analysis of expression of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) (E) and formation of myotube (F) in C2C12 cells transfected with either Wnt5a 3' UTR-WT or Wnt5a 3' UTR-Mut. (G) Western blot analysis of expression levels of Wnt5a in C2C12 cells transfected with Wnt5a 3' UTR. (H–I) Real-time PCR analysis of expression of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) (H) and formation of myotube (I) in C2C12 cells treated Wnt5a siRNA, antagomiR-487b, and combination of above two. $n = 5$ for each group. Each sample was assessed in triplicate. U6 small nuclear RNA is used as the internal control of lncRNA and miRNA. GAPDH is used as the control for mRNA. β -Actin is used as the internal control in western blot. Scale bar = 50 μ m. Data are presented as mean \pm SEM. * $P < 0.05$ vs. corresponding control. # $P < 0.05$ vs. AntagomiR-487b.



binding site for miR-487b (Wnt5a 3' UTR-Mut) (Figure 4B). Luciferase reporter assay revealed that agomiR-487b (agomiR-487b containing wild type binding site for Wnt5a), but not agomiR-487b-Mut (agomiR-487b containing mutated binding site for Wnt5a), could reduce the luciferase activity of Wnt5a 3' UTR. Meanwhile, the luciferase activity of Wnt5a 3' UTR-Mut was not repressed by agomiR-487b. Furthermore, the luciferase activity of Wnt5a 3' UTR was significantly increased after reducing the endogenous levels of miR-487b by treating C2C12 cells with antagomiR-487b (Figure 4C,D). The expression of myogenic markers and formation of myotube were both up-regulated in C2C12 cells by transfection with Wnt5a 3' UTR. The amount of Wnt5a

protein was also markedly elevated in the Wnt5a 3' UTR-transfected C2C12 cells (Figure 4E–G). Moreover, the enhanced expression of myogenic markers and formation of myotube induced by antagomiR-487b treatment was abolished by Wnt5a siRNA (Figure 4H,I).

MAR1 promoted expression level of Wnt5a protein in C2C12 cells

To understand the functional role of MAR1 in Wnt5a regulation, the time course of changes in Wnt5a protein expression was examined in C2C12 cells during differentiation.

It was found that Wnt5a protein was increased during muscle differentiation in C2C12 cells (Figure 5A). The correlation analysis revealed that the expression level of Wnt5a protein was positively associated with MAR1 level in C2C12 cells during differentiation (Figure 5B). Enforced MAR1 expression in C2C12 cells significantly enhanced Wnt5a protein expression, while the elevated Wnt5a protein expression could be abolished by agomiR-487b (Figure 5C). MAR1 shRNA infection decreased Wnt5a protein expression in C2C12 cells, and the reduction of Wnt5a protein expression could be rescued by antagomiR-487b (Figure 5D).

MAR1 promoted skeletal muscle mass/strength and Wnt5a protein level in mice

We further manipulated MAR1 expression in adult mice ($n = 10$) by intramuscular injection of muscle-specific MAR1 overexpression or knockdown lentivirus and sacrificed them 2 months later. Enforced MAR1 expression in mice enhanced gastrocnemius muscle mass and muscle fibre CSA (Figure 6A–D). The muscle also generated significantly higher specific force in the MAR1 overexpressed mice than that in control group (Figure 6E). Moreover, the expression levels of Wnt5a protein, mRNA, and protein of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) were elevated in the muscle from MAR1 overexpressed mice (Figure 6I–L). On the other

hand, MAR1 shRNA infection in gastrocnemius muscle reduced muscle mass and fibre CSA (Figure 6G–J). The specific force generated by muscle was also suppressed after MAR1 shRNA infection (Figure 6M). In addition, MAR1 shRNA infection down-regulated Wnt5a protein, mRNA, and protein levels of myogenic markers in mice muscle (Figure 6N–P).

Enforced MAR1 expression attenuated muscle atrophy in aged mice

To test whether therapeutic enforced MAR1 expression in skeletal muscle could counteract age-related muscle atrophy, 22-month-old mice were intramuscularly injected with muscle-specific MAR1 overexpression lentivirus and sacrificed 2 months afterward ($n = 10$). Compared with baseline and age-matched control group, the expression level of MAR1 in MAR1 overexpression group was notably higher (Figure 7A). Gastrocnemius muscle mass and mean muscle fibre CSA were both reduced in the aged group compared with those in baseline group, while they were enhanced in MAR1 group (Figure 7B–D). Furthermore, we performed *in situ* muscle functional testing. The results showed gastrocnemius muscle in aged group had weaker strength than baseline group, while MAR1 group generated higher force than other two groups (Figure 7E). In addition, the Wnt5a protein level, mRNA, and protein levels of

Figure 5 MAR1 promoted expression level of Wnt5a protein in C2C12 cells. (A) Western blot analysis of expression level of Wnt5a in C2C12 cells during differentiation. $*P < 0.05$ vs. GM. (B) Correlation analysis between expression level of Wnt5a protein and MAR1 in C2C12 cells during differentiation. (C) Western blot analysis of expression level of Wnt5a in C2C12 cells with enhanced MAR1 expression treated with agomiR-487b. (D) Western blot analysis of expression level of Wnt5a in C2C12 cells with inhibited MAR1 expression treated with antagomiR-487b. $n = 5$ for each group. Each sample was assessed in triplicate. U6 small nuclear RNA is used as the internal control of lncRNA and miRNA. GAPDH is used as the control for mRNA. β -actin is used as the internal control in western blot. Data are presented as mean \pm SEM. $*P < 0.05$ vs. control.

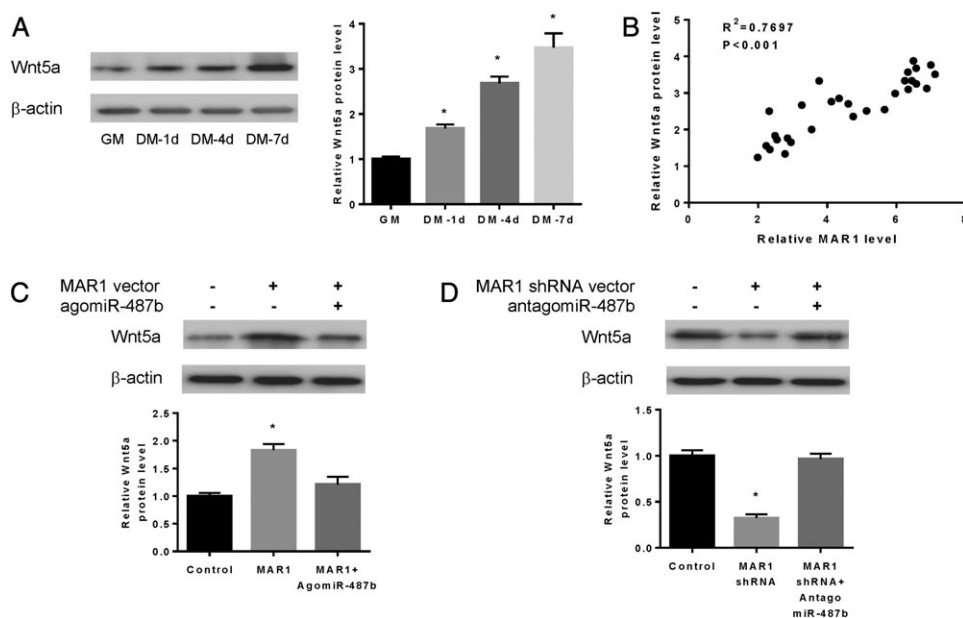
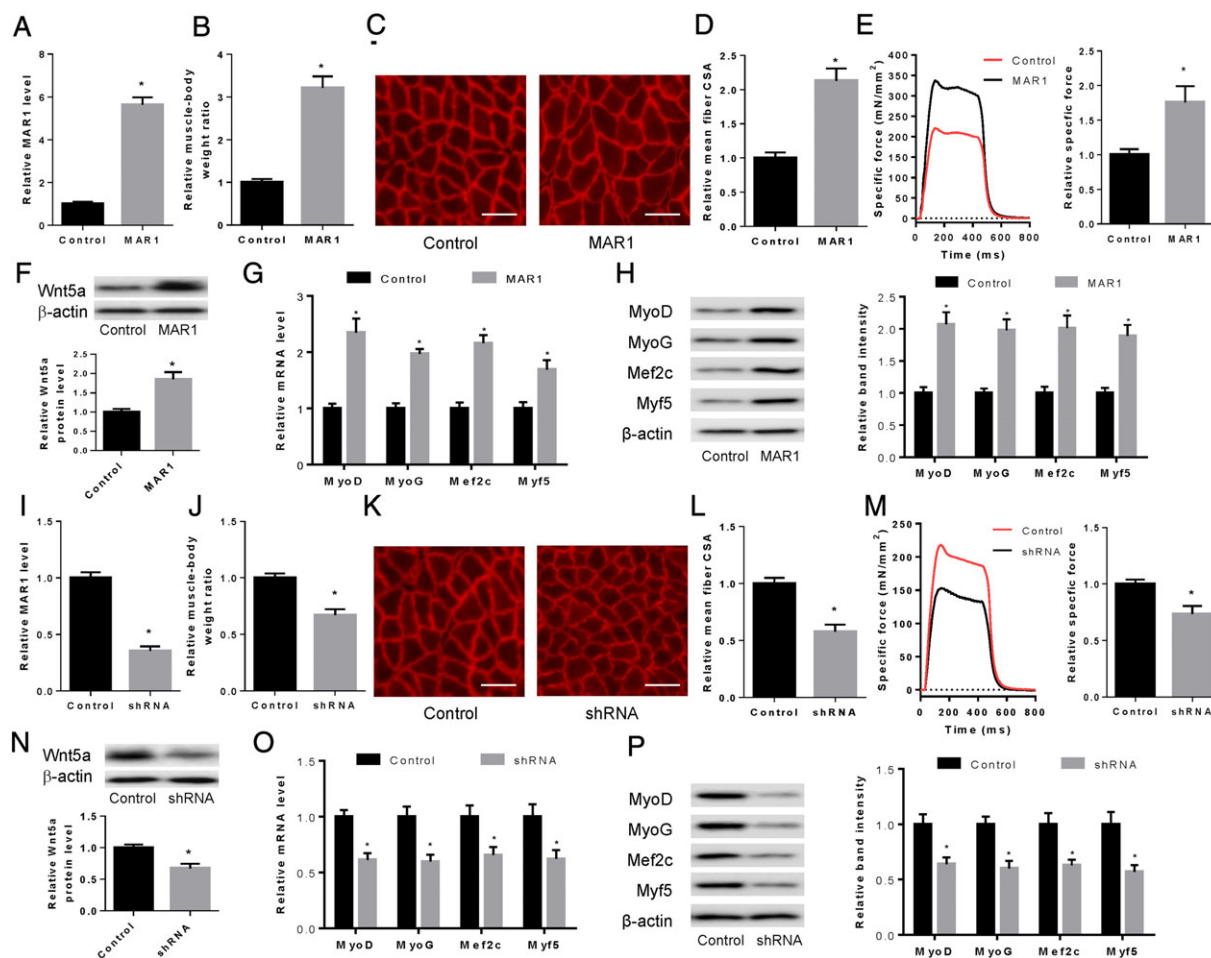


Figure 6 Muscle anabolic regulator 1 (MAR1) promoted skeletal muscle mass/strength and Wnt5a protein level in mice (A) real-time PCR analysis of MAR1 level in gastrocnemius muscle of MAR1 overexpressed mice. (B–D) Gastrocnemius muscle-to-body weight ratio (B), cross-sections from mid-belly gastrocnemius muscle immunostained by anti-dystrophin antibody (C), and mean muscle fibre cross-sectional area (CSA) (D) in MAR1 overexpressed mice. Scale bar = 50 μm . (E) *In situ* muscle function testing of specific force in gastrocnemius muscle of MAR1 overexpressed mice. (F–H) Expression level of Wnt5a protein (F), mRNA (G) and protein levels (H) of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) in gastrocnemius muscle of MAR1 overexpressed mice. (I) Real-time PCR analysis of MAR1 level in gastrocnemius muscle of MAR1 shRNA infected mice. (J–L) Gastrocnemius muscle-to-body weight ratio (J), cross-sections from mid-belly gastrocnemius muscle immunostained by anti-dystrophin antibody (K) and mean muscle fibre CSA (L) in MAR1 shRNA infected mice. Scale bar = 50 μm . (M) *In situ* muscle function testing of specific force in gastrocnemius muscle of MAR1 shRNA infected mice. (N–P) Expression level of Wnt5a protein (N), mRNA (O), and protein levels (P) of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) in gastrocnemius muscle of MAR1 shRNA infected mice. For (D) and (L), the fibre CSA of each muscle was measured from an average of 300 muscle fibres. $n = 10$ for each group. Each sample was assessed in triplicate. U6 small nuclear RNA is used as the internal control of lncRNA and miRNA. GAPDH is used as the control for mRNA. β -Actin is used as the internal control in western blot. Data are presented as mean \pm SEM. * $P < 0.05$ vs. control.



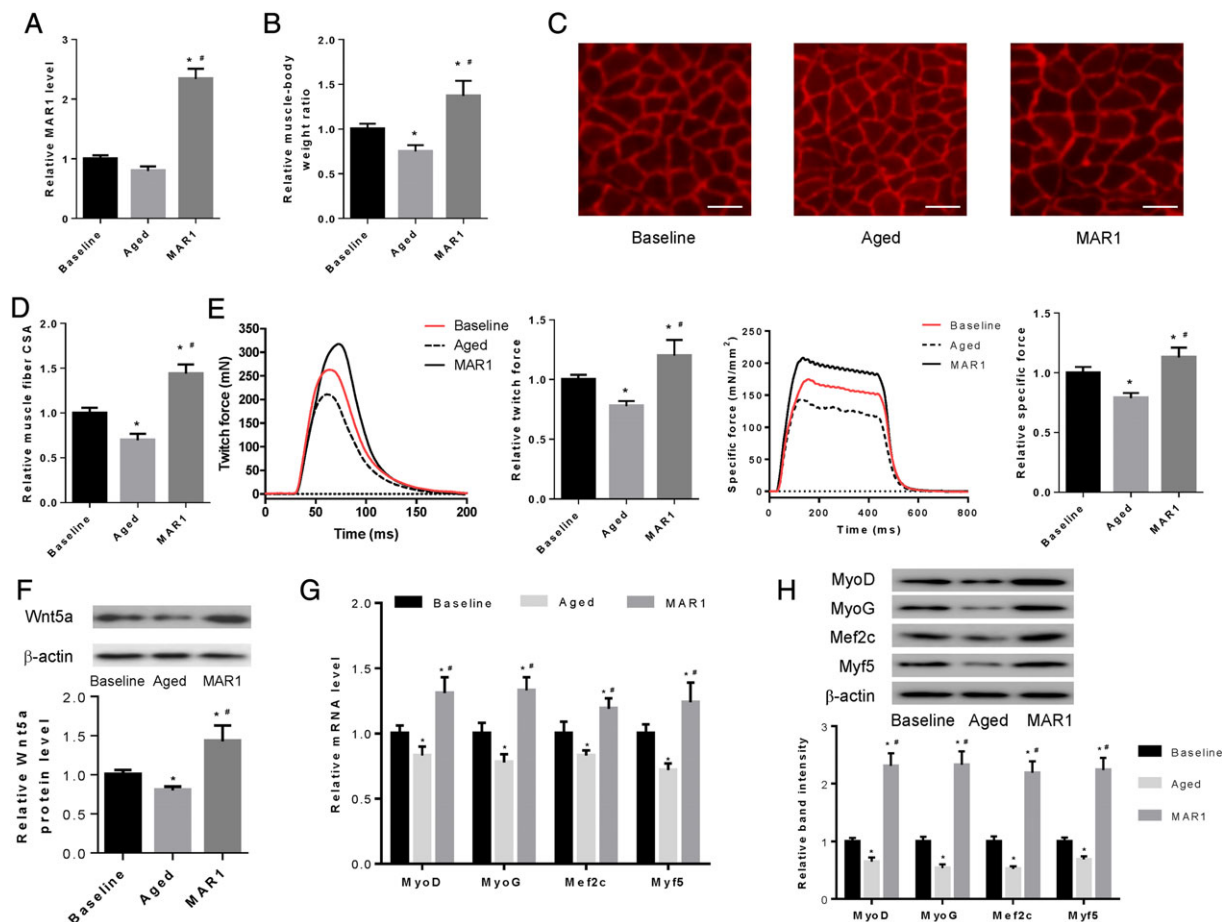
myogenic markers (MyoD, MyoG, Mef2c, and Myf5) showed similar changes with the parameters of muscle mass and strength (Figure 7F–H).

Enforced MAR1 expression attenuated muscle atrophy in hindlimb suspension (HS) mice

To further test whether enforced MAR1 expression in skeletal muscle could counteract mechanical unloading-induced

muscle atrophy, adult mice were intramuscularly injected with muscle-specific MAR1 overexpression lentivirus before 28-day HS and sacrificed when HS finished ($n = 10$). After 28-day HS, MAR1 level in HS group was significantly lower than baseline group, while that in MAR1 group was slightly higher than baseline group (Figure 8A). The muscle mass and mean muscle fibre CSA in MAR1 group were significantly higher than those in HS group, close to the baseline group level (Figure 8B–d). The results of *in situ* muscle functional testing showed that the mechanical unloading-induced

Figure 7 Enforced muscle anabolic regulator 1 (MAR1) expression attenuated muscle atrophy in aged mice. (A) Real-time PCR analysis of MAR1 level in gastrocnemius muscle of aged mice with enforced MAR1 expression. (B–D) Gastrocnemius muscle-to-body weight ratio (B), cross-sections from mid-belly gastrocnemius muscle immunostained by anti-dystrophin antibody (C), and mean muscle fibre CSA (D) in aged mice with enforced MAR1 expression. The fibre CSA of each muscle was measured from an average of 300 muscle fibres. Scale bar = 50 μm . (E) *In situ* muscle function testing of twitch force and specific force in gastrocnemius muscle of aged mice with enforced MAR1 expression. (F–H) Expression level of Wnt5a protein (F), mRNA (G), and protein levels (H) of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) in gastrocnemius muscle of aged mice with enforced MAR1 expression. $n = 10$ for each group. Each sample was assessed in triplicate. U6 small nuclear RNA is used as the internal control of lncRNA and miRNA. GAPDH is used as the control for mRNA. β -Actin is used as the internal control in western blot. Data are presented as mean \pm SEM. * $P < 0.05$ vs. baseline, # $P < 0.05$ vs. aged.



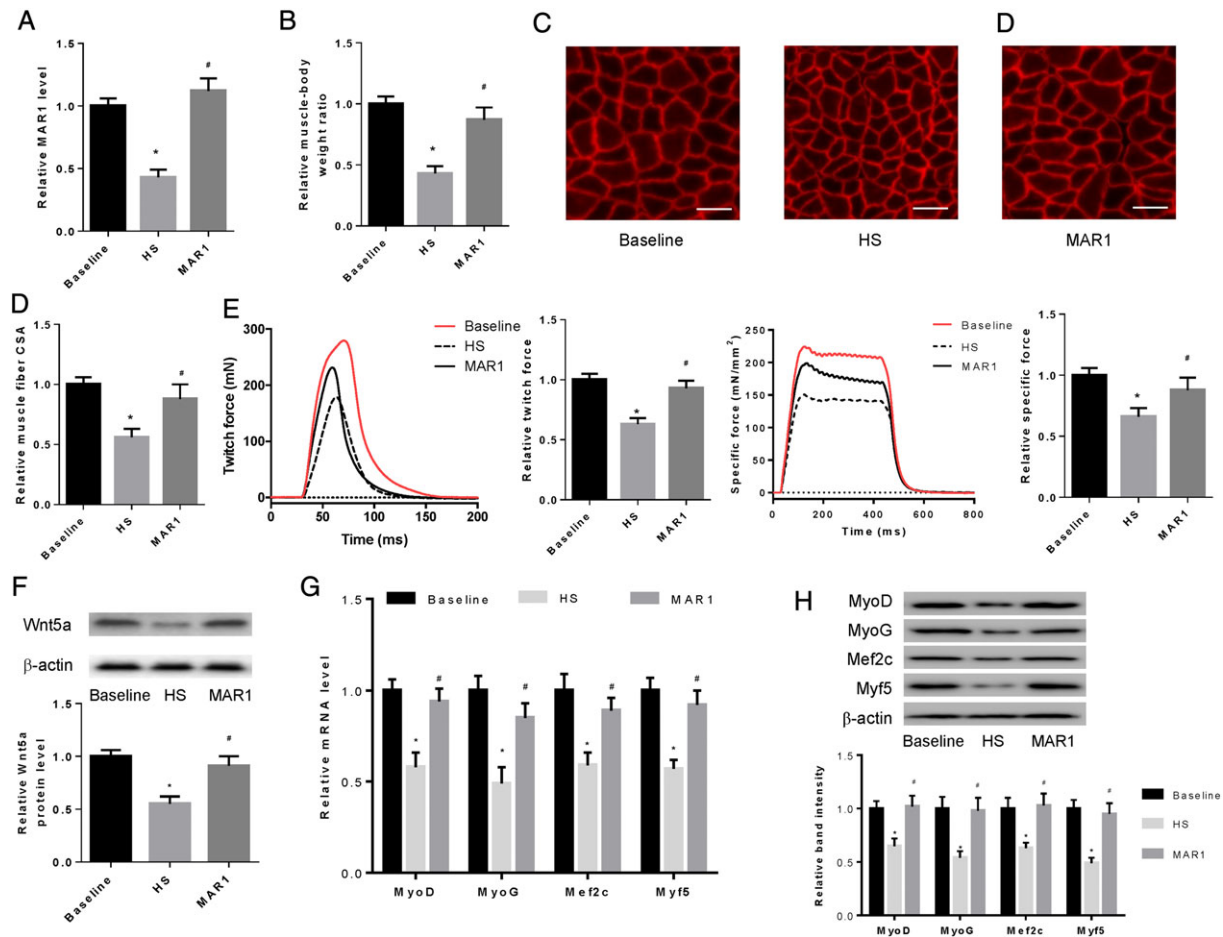
muscle weakness was efficiently attenuated in MAR1 group (Figure 8E). Similarly, the decreased Wnt5a protein level, decreased mRNA, and protein levels of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) were restored in MAR1 group (Figure 8F–H).

Discussion

In this study, we identify a novel lncRNA MAR1 in mice skeletal muscle, functions as a miR-487b sponge to promote skeletal muscle differentiation and regeneration, which could be a novel therapeutic target for treating muscle atrophy induced by either aging or mechanical unloading.

Long non-coding RNAs (lncRNAs) have been demonstrated to regulate muscle differentiation, including H19,^{10,11} linc-MD1,¹² Yam-1,¹³ Malat1,¹⁴ and linc-mg.¹⁵ In current study, the newly identified lncRNA MAR1 was highly expressed in skeletal muscle and associated with the changes of muscle growth during either age-related or mechanical loading-related changes in mice, implying its potential function in regulating muscle differentiation and regeneration. It was further confirmed that MAR1 promoted myogenesis in mice, evidenced by the elevated muscle mass and strength during MAR1 overexpression and the reduced muscle mass and strength when knocking down MAR1, which was consistent with the *in vitro* data. Furthermore, enforced MAR1 expression in skeletal muscle attenuated the reduction of muscle mass and strength in muscle

Figure 8 Enforced muscle anabolic regulator 1 (MAR1) expression attenuated muscle atrophy in hindlimb suspension (HS) mice. (A) Real-time PCR analysis of MAR1 level in gastrocnemius muscle of HS mice with enforced MAR1 expression. (B–D) Gastrocnemius muscle-to-body weight ratio (B), cross-sections from mid-belly gastrocnemius muscle immunostained by anti-dystrophin antibody (C), and mean muscle fiber CSA (D) in HS mice with enforced MAR1 expression. The fibre CSA of each muscle was measured from an average of 300 muscle fibres. Scale bar = 50 μm . (E) *In situ* muscle function testing of twitch force and specific force in gastrocnemius muscle of HS mice with enforced MAR1 expression. (F–H) Expression level of Wnt5a protein (F), mRNA (G), and protein levels (H) of myogenic markers (MyoD, MyoG, Mef2c, and Myf5) in gastrocnemius muscle of HS mice with enforced MAR1 expression. $n = 10$ for each group. Each sample was assessed in triplicate. U6 small nuclear RNA is used as the internal control of lncRNA and miRNA. GAPDH is used as the control for mRNA. β -Actin is used as the internal control in western blot. Data are presented as mean \pm SEM. * $P < 0.05$ vs. baseline, # $P < 0.05$ vs. HS.



atrophy model induced by either aging or mechanical unloading.

Recently, a new regulatory mechanism has been reported whereby lncRNA may function as competing endogenous RNA to sponge miRNA, thereby modulating the derepression of miRNA targets and imposing an additional level of post-transcriptional regulation.³¹ In current study, miR-487b was predicted to contain binding site for MAR1 by bioinformatics analysis. Pull down assay and luciferase assay validated the direct binding of the miR-487b response elements on MAR1 transcript. Furthermore, the enhanced muscle differentiation in MAR1-infected C2C12 cells was attenuated by the wild type miR-487b instead of the mutated miR-487b. MiR-487b has been reported to negatively modulate myoblast

differentiation.³² Wnt5a, an important regulator during myogenesis,^{33,34} has been confirmed to serve as a target of miR-487b in lung cancer cells.³⁰ In our study, luciferase assay validated the binding between miR-487b and Wnt5a 3' UTR in C2C12 cells. Further *in vitro* study confirmed that miR-487b targeted Wnt5a protein to modulate muscle differentiation, evidenced by the enhanced muscle differentiation in C2C12 cells by transfection with Wnt5a 3' UTR and no difference between the treatment of agomiR-487b and antagomiR-487b in C2C12 cells when Wnt5a expression was knocked down. Taken together, when MAR1 is knocked down or overexpressed in C2C12 cells, the expression of miR-487b is up-regulated or down-regulated, resulting in decreased or increased Wnt5a protein level and myogenesis, respectively.

These data indicate that MAR1 may interact with miR-487b to post-transcriptionally regulate the Wnt5a protein.

To date, most studies of lncRNAs in skeletal muscle are investigated in C2C12 cell line or transgenic mice, there has no publication to clarify the role of lncRNA in muscle atrophy animal model.¹⁵ In current study, we overexpressed MAR1 level in skeletal muscle of aged or mechanical unloading mice. MAR1 overexpression attenuated the muscle atrophy induced by either aging or unloading, evidenced by the maintained muscle mass, muscle strength, and the expression levels of myogenic markers. The data show the therapeutic potential of MAR1 in muscle atrophy.

In current study, to achieve skeletal muscle-specific gene expression with high efficiency, we selected mouse α -skeletal actin promoter to construct the lentiviral vectors. The α -skeletal actin plays important roles in skeletal muscle contraction.³⁵ Its promoter has been used for skeletal muscle-specific gene delivery because of its high tissue-specificity and expression efficiency.^{36,37} The intramuscular administration of lentiviral particles used α -skeletal actin promoter that could generate high-level skeletal muscle-specific gene expression for 2 years.³⁷ In our study, direct injection of lentiviral particles constructed with mouse α -skeletal actin promoter into the gastrocnemius muscle of mice resulted in the significantly higher expression level of target gene MAR1 in gastrocnemius muscle from MAR1 lentivirus-treated mice than that in vehicle control mice.

In conclusion, the newly identified lncRNA MAR1 acts as a miR-487b sponge to regulate Wnt5a protein, resulting in promoting muscle differentiation and regeneration. MAR1 could be a novel therapeutic target for treating muscle atrophy induced by either aging or mechanical unloading.

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Competing Financial Interests

The authors declare no competing financial interests.

Online supplementary material

Additional Supporting Information may be found online in the supporting information tab for this article.

Supplementary Table 1. Primer sequences of lncRNA, miRNA and mRNA

Supplementary Figure 1. Schematic of mean muscle fiber cross-sectional area calculation. Six images of different locations in one section were captured at 200x magnification. About Fifty contiguous myofibers in each image were circled to obtain an average of 300 fibers for each muscle. Image J software (National Institutes of Health, Bethesda, MD, USA) were used to determine the area and number of muscle fibers. The mean muscle fiber CSA (μm^2) of each muscle was obtained by dividing the total area of circled muscle fibers in 6 images by the total fiber number in circled area of 6 images.

Supplementary Figure 2. Sequence of MAR1.

Supplementary Table 2. Aberrantly expressed lncRNAs in aged muscle tissues

Supplementary Figure 3. Knockdown efficiency of three shMAR1s

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